Biochemical and Biophysical Research Communications 428 (2012) 105–109

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Regulation of cell motile activity through the different induction of LPA receptors by estrogens in liver epithelial WB-F344 cells

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ARTICLE INFO

Article history:
Received 2 October 2012
Available online 9 October 2012

Keywords:
LPA
LPA receptor
Cell motility
Estrogen

ABSTRACT

Lysophosphatidic acid (LPA) interacts with G protein-coupled transmembrane LPA receptors (LPA receptors; LPA_1–LPA_6). Recently, we demonstrated that each LPA receptor acts as a positive or negative regulator of cell migration ability. It is known that estrogens indicate a variety of biological functions, including cell motility. In the present study, to assess whether LPA signaling is involved in cell motile activity stimulated by estrogens, we measured cell motile activity and LPA receptor expressions of rat liver epithelial WB-F344 cells treated with 17β-estradiol (E_2), ethinyl estradiol (EE) and diethylstilbestrol (DES) at concentrations of 0.1 and 1.0 μM for 48 h. The cell motility of E_2 and EE treated cells was significantly higher than that of untreated cells. By contrast, DES markedly inhibited cell motile activity. Using quantitative real time RT–PCR analysis, *Lpar1* and *Lpar3* expressions in E_2 treated cells were significantly higher than those in untreated cells. In EE treated cells, *Lpar3* expression was markedly elevated, whereas *Lpar1* expression was decreased. On the other hand, *Lpar1* expression was significantly increased in DES treated cells. Interestingly, the effects of E_2, EE and DES on cell motility were suppressed by *Lpar1* or *Lpar3* knockdown. These results suggest that the different induction of LPA receptors by estrogens may regulate cell motile activity of WB-F344 cells.

1. Introduction

Lysophosphatidic acid (LPA) is an extracellular signaling lipid which interacts with G protein-coupled LPA receptors (LPA receptor-1 (LPA_1) to LPA_6) [1–3]. LPA signaling through LPA receptors mediates a variety of cellular functions [1–6]. In cancer cells, LPA receptors also contribute to the acquisition of malignant properties, including cell proliferation, migration, angiogenesis, tumorigenicity and resistance to anticancer drugs [1,7–9]. However, the role of each LPA receptor is not equivalent. For example, LPA_1, LPA_2 and LPA_3 increased cell migration ability of ovarian cancer cells [7]. LPA_3 enhanced cell motility, invasion and tumorigenicity in rat liver tumor cells [8]. In rat neuroblastoma cells, LPA_2 and LPA_3 enhanced cell motility and invasion, but LPA_1 inhibited these activities [9]. By contrast, cell migration activity of rodent lung cancer cells was suppressed by LPA_3 [10].

Sex steroids, estrogens are endogenous hormones and indicate several biological effects, such as cell proliferation, differentiation and cell migration [11,12]. 17β-Estradiol (E_2) is the most potent naturally estrogen in human [13–15]. Ethinyl estradiol (EE) is a common synthetic estrogenic hormone which is used as a contraceptive [13–15]. Diethylstilbestrol (DES) is a synthetic nonsteroidal compound with estrogenic activity [14]. Although DES had been widely used to prevent miscarriage and other complications of pregnancy, it has no longer use because of the possible induction of congenital abnormalities [14]. In addition to several biological functions of estrogens, it is known that the inappropriate actions of estrogens induce the development of cancer cells as the side effects [13].

In the present study, to assess an involvement of LPA signaling on cell motile activity stimulated by estrogens, rat liver epithelial WB-F344 cells were treated with E_2, EE and DES. Then, we measured cell motility and LPA receptor gene expressions of E_2, EE and DES treated cells. Furthermore, we generated LPA receptor knockdown cells from WB-F344 cells and investigated the effects on cell motile activity. Recently, we indicated that 12-O-tetradecanoylphorbol-13-acetate (TPA) stimulated cell migration activity of WB-F344 cells, correlating with the elevated expression level of *Lpar3* [16].

Abbreviations: LPA, lysophosphatidic acid; LPA_1, LPA receptor-1; E_2, 17β-estradiol; EE, ethinyl estradiol; DES, diethylstilbestrol; RT, reverse transcription; PCR, polymerase chain reaction.

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http://dx.doi.org/10.1016/j.bbrc.2012.10.015

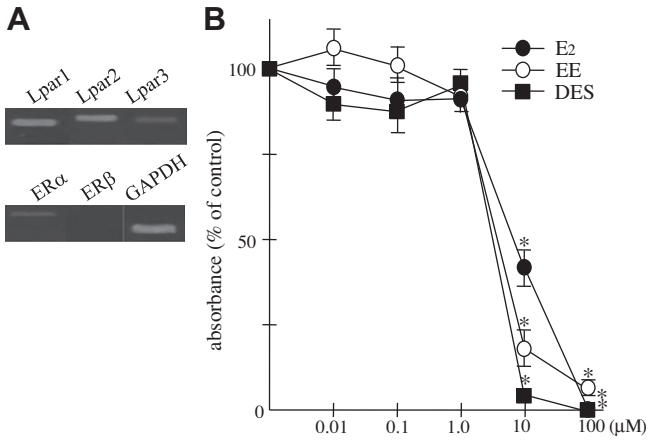

Fig. 1. (A) Semi-quantitative RT–PCR analysis for LPA receptor and estrogen receptor (*ER*) gene expressions in WB-F344 cells. (B) Effects of E_2, EE and DES on cell proliferation rate of WB-F344 cells. Cells were treated with E_2, EE and DES at 0.01, 0.1, 1, 10 and 100 μM for 48 h. Data are indicated as a percentage of untreated (control) cells. Bars indicate SD. $^*p < 0.01$ vs. untreated (control) cells.

Moreover, *Lpar3* knockdown cells markedly suppressed TPA-stimulated cell migration ability [16].

2. Materials and methods

2.1. Cell culture

Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Nacalai Tesque Inc., Kyoto, Japan) containing 10% fetal bovine serum (FBS) in 5% CO_2 atmosphere at 37 °C. *Lpar1* and *Lpar3* knockdown cells (WB-shRNA1-2 and WB-shRNA3-2 cells, respectively) were generated from WB-F344 cells by transfection method of short hairpin RNA (shRNA) for *Lpar1* and *Lpar3* as described previously [16,17].

2.2. RT–PCR analysis

Total RNA was extracted from each cell using ISOGEN (Nippon Gene, Inc., Toyama, Japan), and cDNA was then synthesized from 0.5 μg samples with Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics Co. Ltd., Mannheim, Germany). The gene expression patterns of LPA receptor and estrogen receptor (*ER*) genes were measured by semi-quantitative RT–PCR analysis [8,16]. The amplified products were then separated on 1.5% agarose gels containing 0.05 μg/ml ethidium bromide. For quantitative real time RT–PCR analysis, a Smart Cycler II System (TaKaRa Bio, Inc., Shiga, Japan) and a SYBR Premix Ex Taq (TaKaRa) was also used according to the manufacturer's protocol. The data for *Lpar1* and *Lpar3* genes were normalized to rat *GAPDH* [16,17].

2.3. Effects of E_2, EE and DES on cell proliferation of WB-F344 cells

Cells were plated at 2000 cells/well in a 96-well plate and cultured with 100 μl of DMEM containing 10% FBS. E_2, EE and DES (Sigma Biochemicals, St. Louis, MO, USA) were dissolved in DMSO. Cells were treated with them at a concentration of 0.01, 0.1, 1.0, 10 and 100 μM per dish for 3 days. E_2, EE and DES were added every 24 h. To measure the effects on cell growth, solution from a Cell Counting Kit-8 (CCK-8) (Dojin Chemistry, Kumamoto, Japan) was added to the plate at 3 days and cells were further incubated for 1 h. The absorbance of the culture medium at 450 nm was determined. The assay was always done in triplicate [8,9,16–18].

2.4. Effect of E_2, EE and DES on cell motile activity of WB-F344 cells

To assess the effects of E_2, EE and DES on cell motile activity of WB-F344 cells, a Cell Culture Insert (BD Falcon, NJ, USA) with 8 μm pore size was used. Cells were pretreated with E_2, EE and DES at a concentration of 0.1 and 1.0 μM for 48 h, and were seeded in the filter at 1×10^5 cells in 200 μl serum-free DMEM (upper chamber) and placed in 24-well plates (lower chamber) containing 800 μl of DMEM containing 10% FBS. Cells were incubated for 24 h in 5% CO_2 atmosphere at 37 °C. Cells remaining in the upper side of the filter were removed with cotton swabs. After Giemsa staining, the number of cells migrated to the lower side of the filter was counted. Each experiment was repeated three times [8,9,16,17].

3. Results and discussion

In our recent study, we demonstrated that TPA which is a tumor promoting agent stimulated cell motile activity of WB-F34 cells its activity was significantly inhibited by *Lpar3* knockdown [16]. In this study, to evaluate an involvement of LPA receptors on cell motility

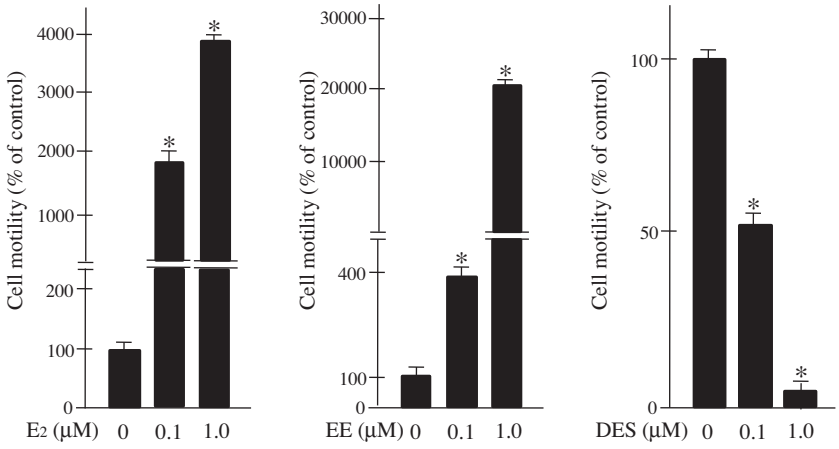

Fig. 2. The cell motility assay with a Cell Culture Insert. Cells were pretreated with E_2, EE and DES at a concentration of 0.1 and 1.0 μM for 48 h, and were seeded in the filter at 1×10^5 cells in 200 μl serum-free DMEM (upper chamber) and placed in 24-well plates (lower chamber) containing 800 μl of DMEM containing 10% FBS. Columns indicate the mean of three studies. Bars indicate SD. $^*p < 0.01$ vs. untreated (control) cells.